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1314576

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APPLICATION NUMBER: ***60/632,456***
FILING DATE: ***December 02, 2004***
RELATED PCT APPLICATION NUMBER: ***PCT/US05/03183***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV440868563US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
John P.	Toscano	Baltimore, MD
Preeya	Kapur	Frederick, MD

Additional inventors are being named on the ______ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

NEW NITROXYL DONORS

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number:

OR

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Country	USA	Telephone	410-516-8300	Fax	410-516-5113

ENCLOSED APPLICATION PARTS ***(check all that apply)***

☑ Specification *Number of Pages* 29
☐ Drawing(s) *Number of Sheets* ______
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number ______
☐ Other (specify) ______

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees.
☐ The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: ______
☑ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☒ Yes, the name of the U.S. Government agency and the Government contract number are: GM58109

Respectfully submitted,

SIGNATURE [signature]

TYPED or PRINTED NAME Gregory Schreiber, Ph.D

TELEPHONE 410-516-8300

Date 02-DEC-04

REGISTRATION NO. 55,601
(if appropriate)
Docket Number: 4390

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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I hereby certify that this correspondence (along with any papers referred to as being attached or enclosed) is being deposited with the United States Postal Service as Express Mail, Post Office to Addressee with sufficient postage in a **Flat Rate** envelope addressed to MS Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date indicated below:

02-DEC-04
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And of Mail Deposit

Signature

JHU Ref: 4390

United States Provisional Patent Application

NEW NITROXYL DONORS

by

John P. Toscano

and

Preeya Kapur

Johns Hopkins University
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NOVEL NITROXYL DONORS

This invention was made with Government support under gm-58109 awarded by the PHS. The Government has certain rights in the invention.

All references cited in this provisional patent application are herein incorporated by reference, each in its respective entirety.

Johns Hopkins University LTD
Licensing & Technology Development

RECEIVED
LICENSING & TECHNOLOGY DEVELOPMENT

Report of Invention Disclosure Form (ROI)

This form is to be completed and submitted to the JHU office of Licensing and Technology Development (LTD) by anyone who believes they have developed a new invention. The purpose of this form is to enable LTD to evaluate whether legal protection to the invention will be sought and/or commercialization pursued. Please submit this form with all inventor(s) and Department Director(s) signatures. Visit the LTD web site at http://jhu.edu/technology/roi.html for HTML and Word downloadable formats of this form.

INVENTION INFORMATION

Title of Invention: [Title should be sufficiently descriptive to identify the invention yet not reveal unique unpublished details.]

New Nitroxyl Donors

Name of Lead Inventor: Toscano, John P., Ph.D.

Last First Middle Degree

Lead Inventor Information: [The Lead Inventor is the primary contact person for LTD on all matters associated with this Report of Invention, including processing, patent prosecution and licensing. For reasons of administrative efficiency, it is the responsibility of the Lead Inventor to keep all other JHU inventors named on this Report of Invention informed of the status of such matters.]

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Business address: Department of Chemistry, Johns Hopkins University
3400 N. Charles Street, Baltimore, MD 21218

Interdepartmental address: Department of Chemistry

Home phone number: (410) 372 - 0562 Home fax number: ( ) -

Home address: 709 Regester Avenue, Baltimore, MD 21212

Citizenship: USA Social Security Number:

Are you a Howard Hughes Medical Institute employee or investigator? ☐ Yes ☒ No
Are you a Kennedy Krieger Institute employee or investigator? ☐ Yes ☒ No

Additional inventors: ☐ Yes ☐ No If yes, please complete Additional Inventors section for each inventor.

LTD Internal Use Only: **REF-** 4390 TLA GHS Field of Use 2C

3

JHU Ref: 4390

ADDITIONAL INVENTION INFORMATION
Please copy this page for additional inventors as necessary

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Are you a Kennedy Krieger Institute employee or investigator?	☐ Yes ☒ No

Name of Inventor: Last First Middle Degree	
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School:	Department:
Business phone: ( ) -	Business fax: ( ) -
Business address: Interdepartmental address:	
Home phone number: ( ) -	Home fax number: ( ) -
Home address:	
Citizenship:	Social Security Number:
Are you a Howard Hughes Medical Institute employee or investigator?	☐ Yes ☐ No
Are you a Kennedy Krieger Institute employee or investigator?	☐ Yes ☐ No

INVENTION DESCRIPTION

Describe the invention completely, using the outline given below. Please provide an **electronic copy** of the invention disclosure document, references, and abstracts in Windows format on CD-ROM or floppy disk if possible

1. **Marketing Summary** [Please provide a non-confidential summary of the invention that can be used for marketing purposes. Unique details that are published may also be included.]

New nitroxyl (NO^-/HNO) donors have been developed based on diazen-1-ium-1,2-diolate derivatives ($R^1R^2N[N(O)=NO]Na$). Such derivatives normally decompose under physiologically relevant conditions to amine (R^1R^2NH) and nitric oxide (NO). These newly developed derivatives, however, give nitrosamine ($R^1R^2NN=O$) and nitroxyl. These new nitroxyl precursors have been shown to have analogous effects in the treatment of heart failure as has previously been observed with the established nitroxyl donor Angeli's salt.

SOFTWARE –Does this disclosure include a software element or software is implemented in the invention ☐ Yes ☒ No
If yes, please complete the Software Information Form which can be found at: ______
BIOLOGICAL MATERIAL – Does this disclosure include biological material, ☐ Yes ☒ No
If yes, please attach a list of materials for reference. A Tangible Property Report of Invention form may be completed if the disclosure is biological materials only. You can find this form at: http://www.hopkinsmedicine.org/lbd/otl/

2. **Problem Solved** [Describe the problem solved by this invention]

Most importantly, these new nitroxyl precursors are novel compounds. In addition, almost all previous physiological studies probing the effects of nitroxyl have used Angeli's salt, which decomposes with a half-life of approximately 2 minutes. A potential reaction pathway for nitroxyl is dimerization to provide ultimately nitrous oxide (N_2O) and water. Because this second-order reaction is dependent on the local concentration of nitroxyl, the rate at which nitroxyl is produced determines what portion of it is available for other chemistry, i.e., faster decomposition rates lead to more dimerization. Our newly developed compounds have half-lives of approximately 12 minutes. Moreover, this half-life may potentially be varied by changing R^1 and/or R^2. Thus, studies with these new precursors (and analogous derivatives) will help to determine if biological responses due to nitroxyl can be enhanced (or retarded) by its delivery rate.

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3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]

The compounds themselves are novel.

4. Potential Commercial Use – [What products can be produced with this invention.]

The administration of a nitroxyl-donating compound either alone, in combination with a positive inotropic agent, or to a subject receiving beta-antagonist therapy can be used to treat heart failure of all classifications. In particular, a nitroxyl-donating compound can be used to treat early-stage chronic heart failure, such as Class II heart failure. Potentially, nitroxyl-donating compounds can be used also in subjects suffering from hypertension.

5. Commercialization - List any companies that you feel may be interested in this technology or are doing similar research. Indicate how the invention complements the company's existing technology. If known, provide the names of any companies (and a contact person) that have contacted you regarding your research related to the invention.

☒ No company interest known at this time.

Keywords – Please circle the categories and keywords that accurately describe the present invention.

CHEMICAL
☐ Additives
☐ Alternative Energy
☐ Antioxidants
☐ Batteries
☐ Catalyst
☐ Coal Conversion
☐ Coatings
☐ Effluent Treatment
☐ Elastimers
☐ Electrochemistry
☐ Exhaust Treatment
☐ Foams
__ Food Chemistry
__ Fuel Cells
__ Gas Conversion
__ Gels
__ Monomers
__ Oxidation
__ Petroleum
__ Photochemistry
__ Polymers
__ Remediation
__ Solvents

DIAGNOSTIC
__ Antibody
__ Assay
__ Biochip
__ Contrast Agent
__ Detection
__ DNA Probe
__ Elisa
__ Imaging
__ Immunoassay
__ In Situ
__ Marker
__ Measurement
__ MRI
__ Point of Use
__ Radioisotope
__ Transgenic
__ Ultrasound

GENOMICS
__ Allele
__ Bioinformatic
__ cDNA
__ Epidemiology
__ EST
__ Gene
__ Homologue
__ Isogene
__ Library
__ Mutation
__ Pharmacogenomics
__ Polymorphism
__ Positional Cloning
__ Proteomics
__ Receptor
__ RNA
__ Target Validation

MEDICAL DEVICE
__ Delivery
__ Diagnosis
__ Imaging
__ Measurement
__ Optical
__ Safety
__ Surgical
X Treatment

RESEARCH TOOL
__ Animal Model
__ Antibody
__ Cell Line
__ Culture
__ Directed Evolution
__ DNA Probe
__ DNA/RNA Sequencing
__ DNA/RNA Synthesis
__ Electrophoresis
__ Elisa
__ Enzyme
__ Equipment
__ Expression System
__ Immunoassay
__ Label
__ PCR
__ Protein Sequencing
__ Protein Synthesis
__ Reagent
__ Spectroscopy
__ Tissue Culture
__ Vector

SCREENING
__ Assay
__ Biochip
__ Combinatorial Biology
__ Combinatorial Chemistry
__ Detection
__ HTS
__ Phage Display
__ Screen
__ Target

THERAPEUTIC
__ Analgesic
__ Anesthetic
__ Angiogenesis
__ Antibiotic
__ Antibody
__ Antifungal
__ Antiinflammatory
__ Antisense
__ Antiviral
__ Apoptosis
__ Cell Signaling
__ Cell Therapy
__ Disease Model
X Drug Delivery
X Drug Design
__ Fertility
__ Gene Therapy
__ Hormone
__ Immunotherapy
__ Natural Product
__ Peptides
__ Pro-drug
__ Proteins
__ Small Molecule
__ Tissue Engineering
__ Transplant
__ Vaccine
__ Virus
__ Wound Healing

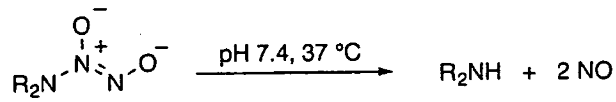
DISEASES
__ Aging
__ Blood
__ Cancer
X Cardiovascular
__ Dermatologic
__ Endocrine
__ Gastrointestinal
__ Genitourinary
__ Hepatic
__ Immune
__ Infectious
__ Metabolic
__ Musculoskeletal
__ Neurological
__ ObGyn
__ Ophthalmological
__ Oral
__ Pediatric
__ Psychiatric
__ Respiratory

ADDITIONAL KEY WORDS:
____
____
____

STAGE OF DEVELOPMENT
__ Unspecified
__ Discovery
__ Preclinical
__ Prototype
__ Phase I
__ Phase II
__ Phase III
__ NCE

Detailed Description of the Invention

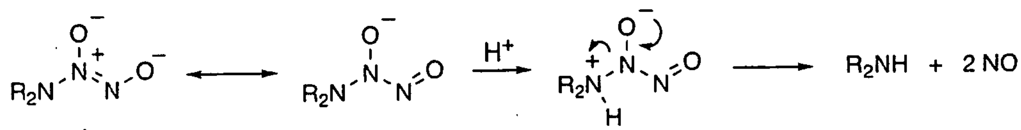
Compounds containing the diazeniumdiolate [N(O)=NO]$^-$ functional group have proven useful as research tools in a variety of applications requiring spontaneous release of nitric oxide (NO).[1] Anions such as 1-(*N,N*-dialkylamino)diazen-1-ium-1,2-diolates **1** (where R is alkyl) are stable as solid salts, but release up to 2 mol of NO when dissolved in aqueous solution at physiologically relevant conditions.



1

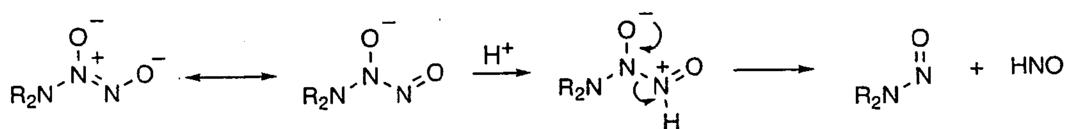
The formation of such compounds by the reaction of NO with nucleophiles such as amines has been known since the 1960's.[2-5] More recently, Keefer and co-workers have shown that the rate of NO release can be varied by modifying the substituents R, pH, or temperature, and have developed anions with half-lives in aqueous buffer at pH 7.4 and 37 °C ranging from two seconds to 20 hours.[1] In addition, diazeniumdiolate solution half-lives tend to correlate very well with their pharmacological durations of action, suggesting that they are minimally affected by metabolism.[6] These compounds have shown great potential in a variety of medical applications requiring either the rapid production or gradual release of NO,[6,7] and have allowed biological consequences of NO delivery rates to be probed.[8]

A major factor affecting decomposition rate is ease of protonation at the amine nitrogen leading to amine and 2 equivalents of NO:



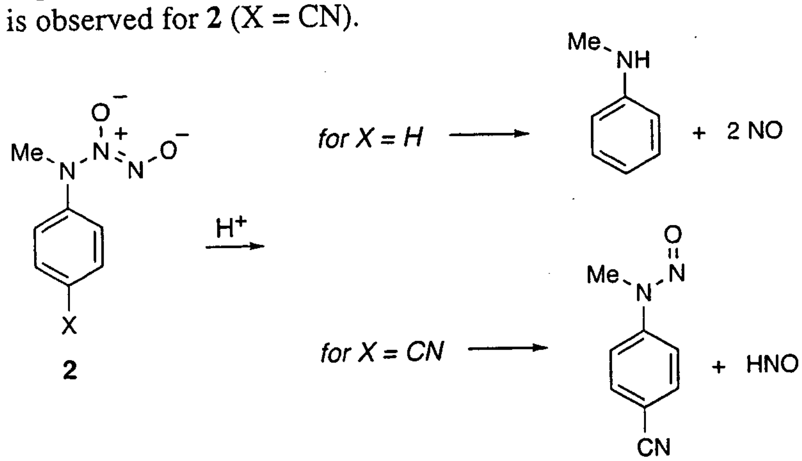
1

We reasoned that if protonation at this site was made very unfavorable that an alternate decomposition pathway to nitrosamine and nitroxyl (NO$^-$/HNO) may become available:



1

Thus, we observe completely different decomposition products for the related *N*-methylaniline derivatives **2** with X = H or CN. For the parent compound **2** (X = H) we observe the normal decomposition to amine and NO with a half-life of approximately 4 minutes at pH 7.4 and 37 °C. With an electron-withdrawing substituent, however, protonation at the aniline nitrogen becomes very unfavorable and decomposition to nitrosamine and nitroxyl, with a half-life of approximately 12 minutes at pH 7.4 and 37 °C, is observed for **2** (X = CN).



2

Compound A

Compound B

JHU Ref: 4390

Each of these compounds has been tested for their effects on cardiac function in canine models. In agreement with the observed products, **2** (X = H) behaves as an NO-donor, whereas **2** (X = CN) behaves as a nitroxyl-donor. We believe that compound **2** (X = CN) and analogous derivatives (described in the following Workable Extent/Scope section) have great potential in the treatment of heart failure.

Synthetic Procedure: Compounds **2** were prepared by treating a solution of the appropriate *N*-methylaniline derivative (1 g) in methanol (5 mL) with one equivalent of sodium methoxide (25 % w/w in methanol) in a standard Parr hydrogenation bottle. The reaction vessel was purged with nitrogen and then saturated with excess NO. The reaction was allowed to stir at room temperature for 48 hours during which time the pressure of NO gas was maintained at approximately 40 psi. The product was isolated by filtration and washed with ethyl ether and dried under vacuum. Half-lives were determined by UV-Vis spectroscopy at 37 °C in pH 7.4 phosphate buffer. NO was detected electrochemically using an iNO Measuring System with an amino 700 probe (Innovative Instruments). Nitroxyl was measured by trapping with methemoglobin as has been described in the literature.[9]

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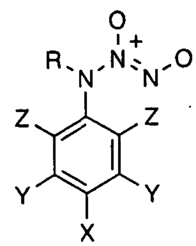
Workable Extent/Scope

Our results obtained to date are easily extendable to related derivatives that can be expected to follow the same decomposition pathway to nitrosamine and nitroxyl. Obvious examples are listed below. Another issue that will require further research is related to the nitrosamine byproduct. Although many nitrosamines are carcinogenic, the extent of carcinogenicity can be greatly reduced or eliminated by blocking sites for enzymatic hydroxylation, the key activation step leading to subsequent DNA alkylation (e.g., by substitution at the carbon alpha to the *N*-nitroso functionality or by carboxylic acid substitution).[10] The toxicity of the nitrosamine derived from **2** (X = CN) is not yet known, but it is not expected to be high based on related nitrosamines that have been reported in the literature.[11]

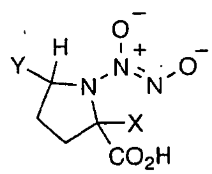
Other *N-Methylaniline Derivatives*



where R is H, a primary, secondary, or tertiary alkyl group, or an aromatic group; X is an electron-withdrawing substituent (e.g., halogen, CN, NO_2, CO_2H, CO_2R, CF_3); Z is H, an alkyl group, or an electron-withdrawing substituent (e.g., halogen, CN, NO_2, CO_2H, CO_2R, CF_3); Y is H or CO_2H.

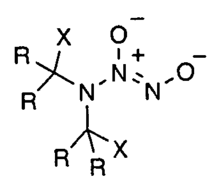
Other *Proline Derivatives*

(*N*-nitrosoproline is known to be non-carcinogenic.)



where X is a halogen and Y is an H or halogen.

Other *Diethylamine Derivatives*



where R is an H or alkyl group and X is an electron-withdrawing group (e.g., halogen, CN, NO_2, CO_2H, CO_2R, CF_3).

JHu Ref: 4390

References

(1) Hrabie, J. A.; Keefer, L. K. *Chem. Rev.* **2002**, *102*, 1135-1154.

(2) Drago, R. S.; Karstetter, B. R. *J. Am. Chem. Soc.* **1960**, *83*, 1819-1822.

(3) Drago, R. S.; Paulik, F. E. *J. Am. Chem. Soc.* **1960**, *82*, 96-98.

(4) Drago, R. S.; Ragsdale, R. O.; Eyman, D. P. *J. Am. Chem. Soc.* **1961**, *83*, 4337-4339.

(5) Longhi, R.; Ragsdale, R. O.; Drago, R. S. *Inorg. Chem.* **1962**, *1*, 768-770.

(6) Keefer, L. K. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 585-607.

(7) Saavedra, J. E.; Fitzhugh, A. L.; Keefer, L. K. *Nitric Oxide and the Cardiovascular System* **2000**, 431-446.

(8) Mooradian, D. L.; Hutsell, T. C.; Keefer, L. K. *J. Cardiovasc. Pharmacol.* **1995**, *25*, 674-678.

(9) (a) Addison, A. W.; Stephanos, J. J. *Biochemistry*, **1986**, *25*, 4104-4113. (b) Bazylinski, D. A.; Hollocher, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 7982-7986.

(10) Lijinsky, W. *Chemistry and Biology of N-Nitroso Compounds*, Cambridge University Press: Cambridge, UK, 1992.

(11) (a) Guo Z.; McGill A.; Yu L.; Li, J.; Ramirez, J.; Wang P. G. *Bioorg. Med. Chem. Lett* **1996**, *6*, 573-578. (b) Guo Z.; Xian M.; Zang, W.; McGill A.; Wang P. G. *Bioorg. Med. Chem. Lett.* **2001**, *9*, 99-106.

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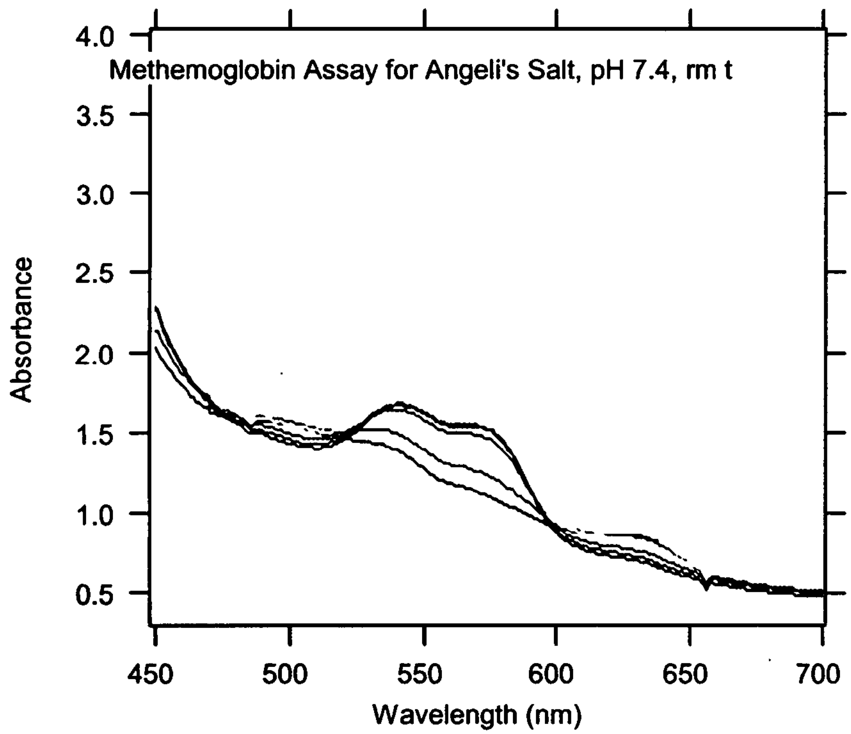
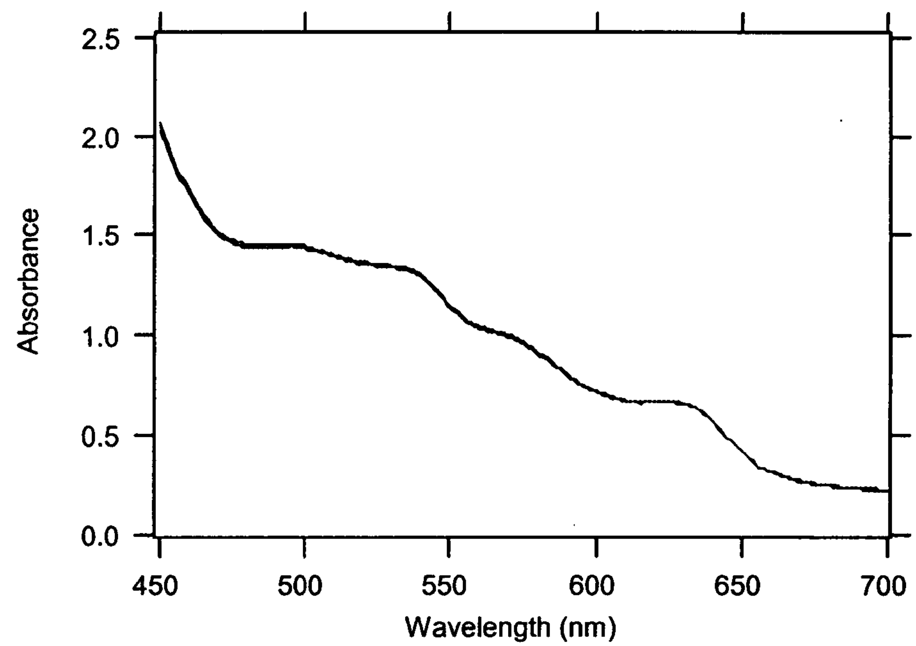
Quenching Methemoglobin Assays with Glutathione for Angeli's Salt

Glutathione reacts with HNO faster than Fe(III) reacts with HNO, therefore it is a good indicator of whether or not the Fe(II)-NO signal (seen on the left) is from HNO or some other reaction pathway. Loss of any growth around the 520-580 nm (seen on the right) region indicates quenching of the reaction

In the absence



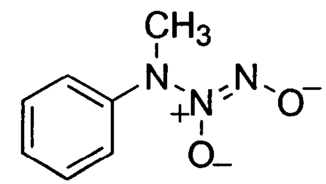
In the presence

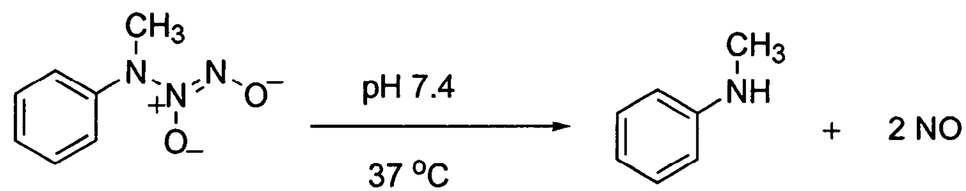


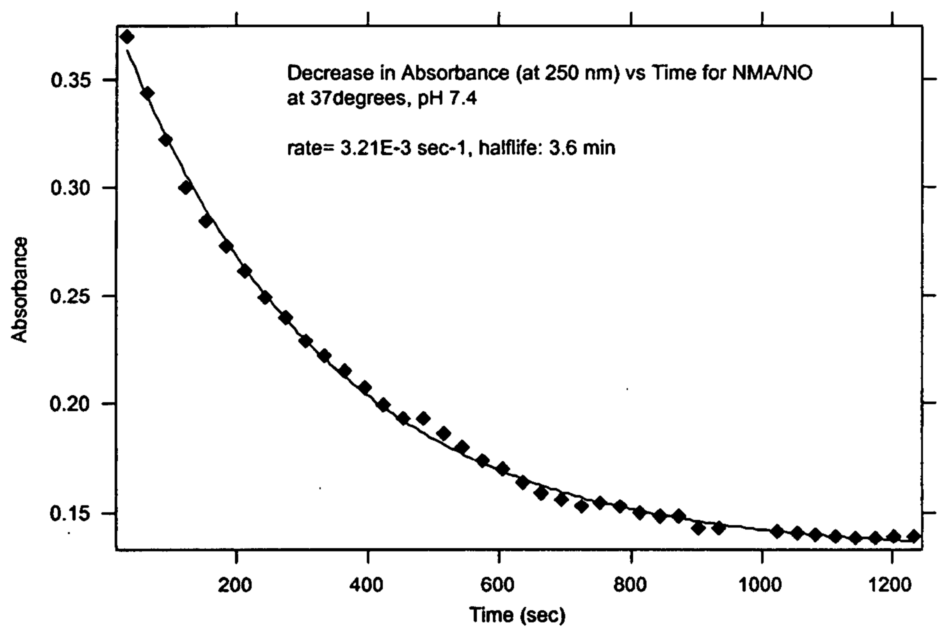
(left), 50uM Methemoglobin, 100 uM HNO donor, pH 7.4 50mM phosphate buffer; (right) same with added 1mM glutathione

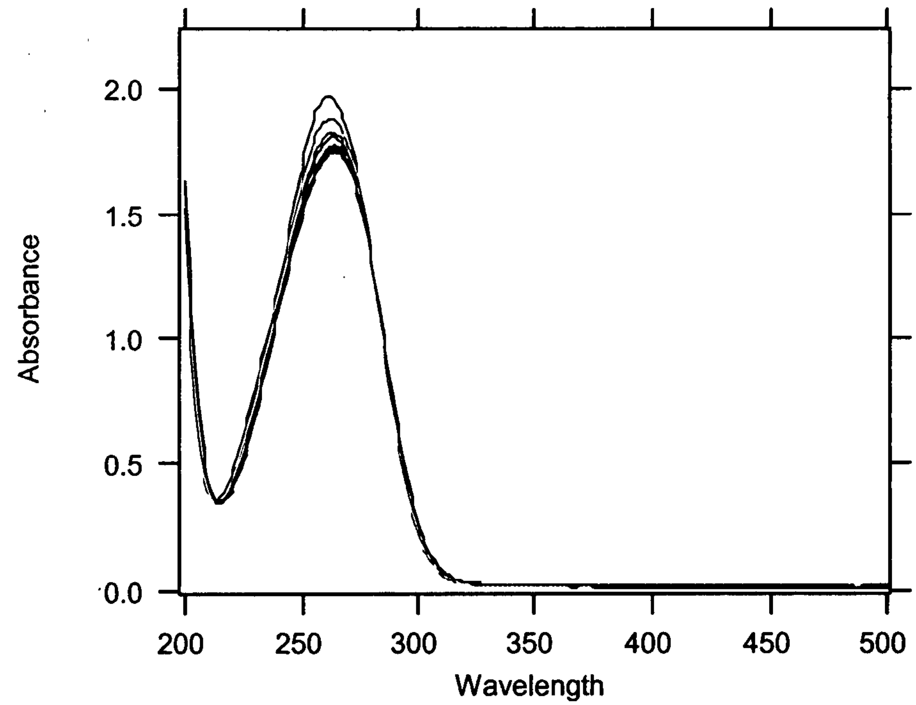
12

JHu Ref: 4390

Thermal Decomposition of: 

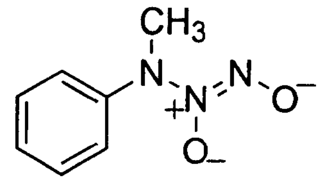


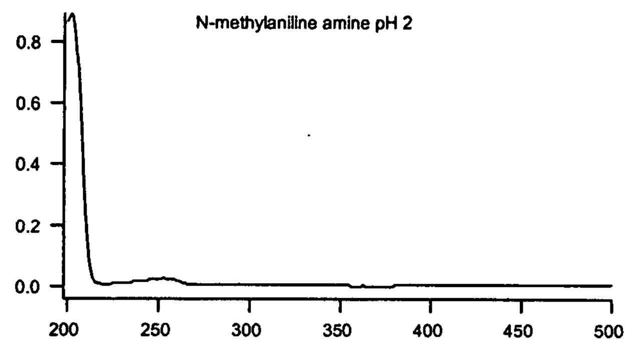




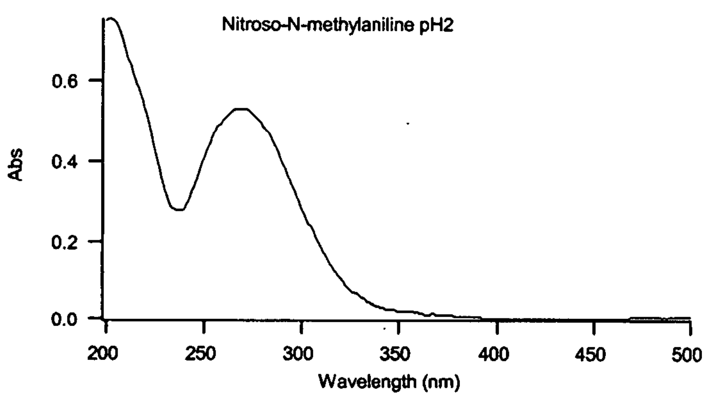
(left): Kinetics of decomposition at 37 degrees C, pH7.4, monitored at 250 nm (max absorbance of NO donor). (right): spectral data of the decay taken over a period of 1 hour.

JHu Ref: 4390

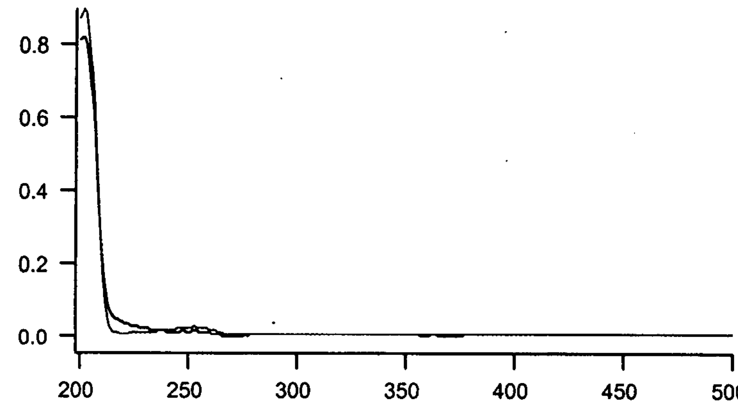
Decomposition Assay of 



A. *N-methylaniline UV spectrum at pH2*



B. *N-Nitroso-N-methylaniline UV spectrum at pH2,*

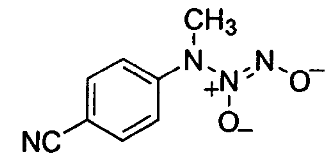


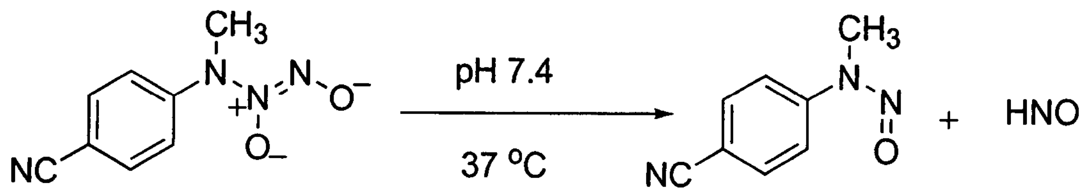
C. *Product of Decomposition at pH2 in an anaerobic environment. In red is the overlay of N-methyl aniline UV spectrum at pH2.*

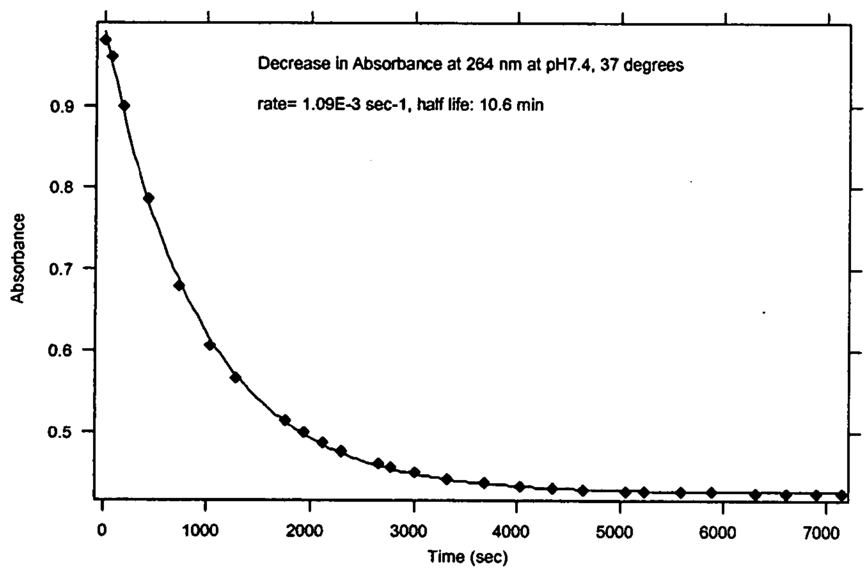
This assay shows that no nitrosamine is formed during decomposition, nitrosamine is a product of the nitrosamine/HNO complexes, not amine/NO complexes under these conditions.

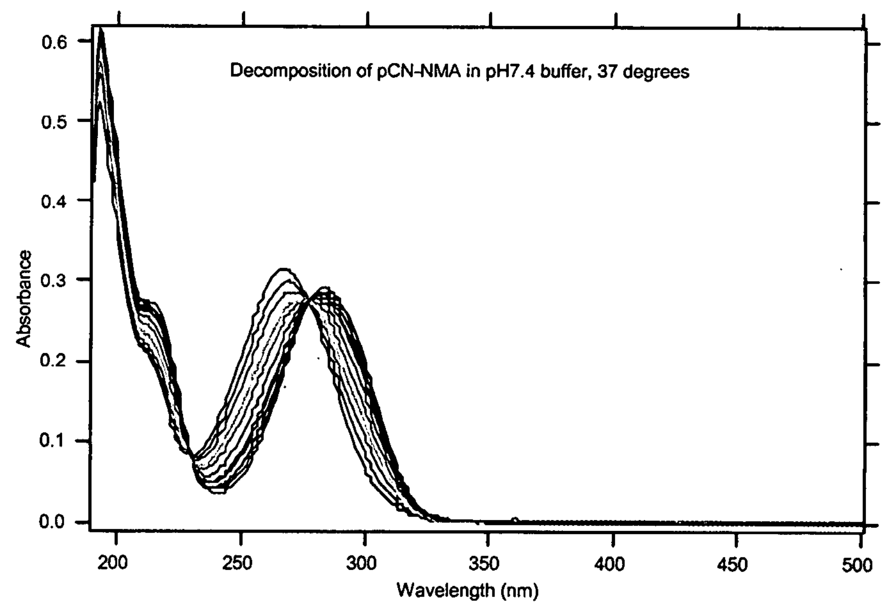
JHu Ref: 4390

Thermal Decomposition of:









(left): Kinetics of decomposition at 37 degrees C, pH7.4, monitored at 264 nm (max absorbance of HNO donor). (right): spectral data of the decay taken over a period of 2 hours.

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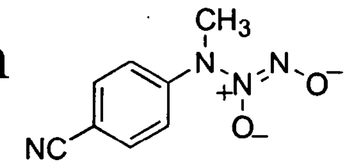
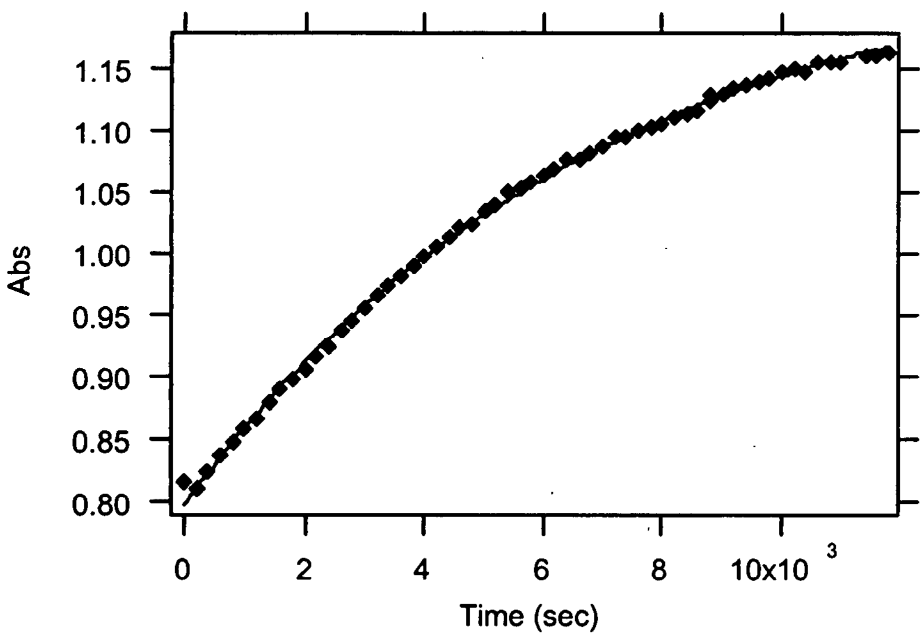
JHu Ref. 4390

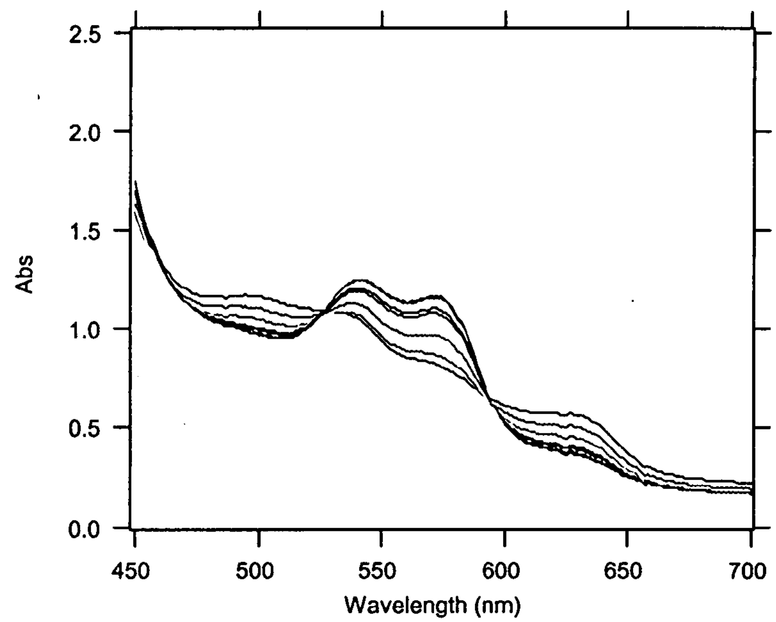
Methemoglobin (Hb^+) Assays with 

$$Fe(III) + HNO \longrightarrow Fe(II)\text{-}NO$$

Kinetics of Hb^+ binding to HNO



Spectral Monitoring of Hb^+ binding to HNO



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(left): Kinetics of Fe(II)-NO production at pH7.4, monitored at 572 nm, concentration of HNO donor: 100 uM and Methemoglobin 50 uM; The change in absorbance at 572 nm (E=13,000 $M^{-1}cm^{-1}$) is equal to 1 eq of HNO (right): spectral data taken over a period of 2 hours.

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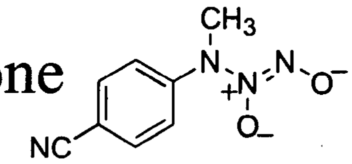
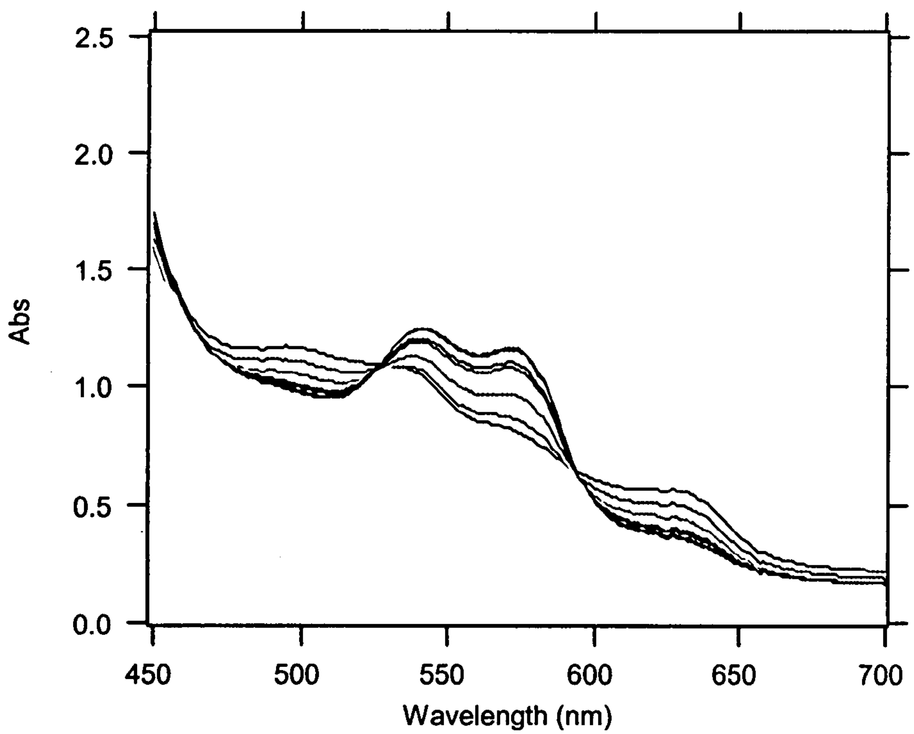
Quenching Methemoglobin Assays with Glutathione

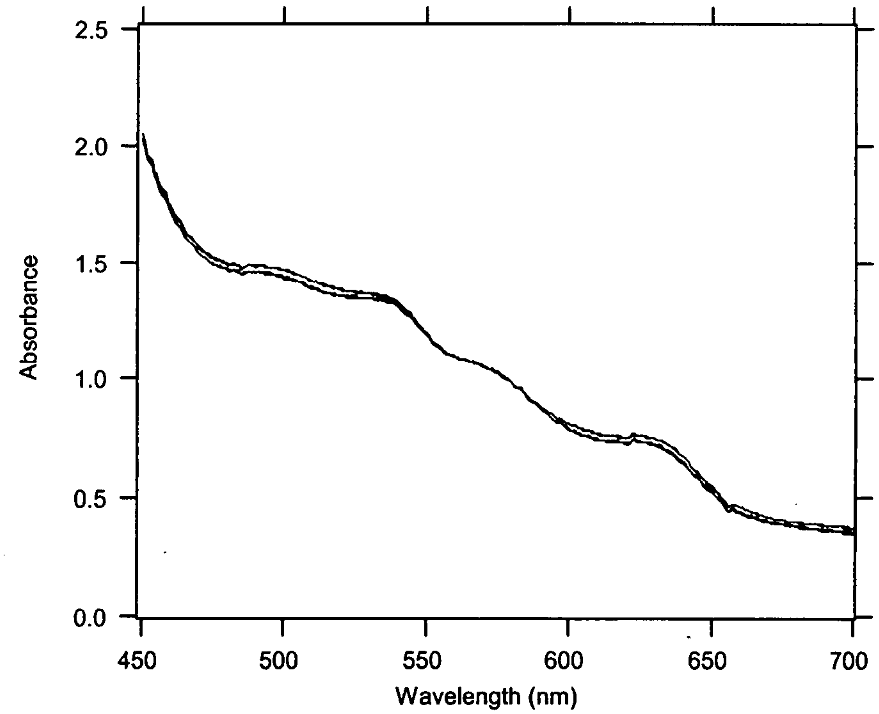


Glutathione reacts with HNO faster than Fe(III) reacts with HNO, therefore it is a good indicator of whether or not the Fe(II)-NO signal (seen on the left) is from HNO or some other reaction pathway. Loss of any growth around the 520-580 nm (seen on the right) region indicates quenching of the reaction

In the absence



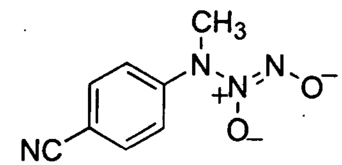
In the presence

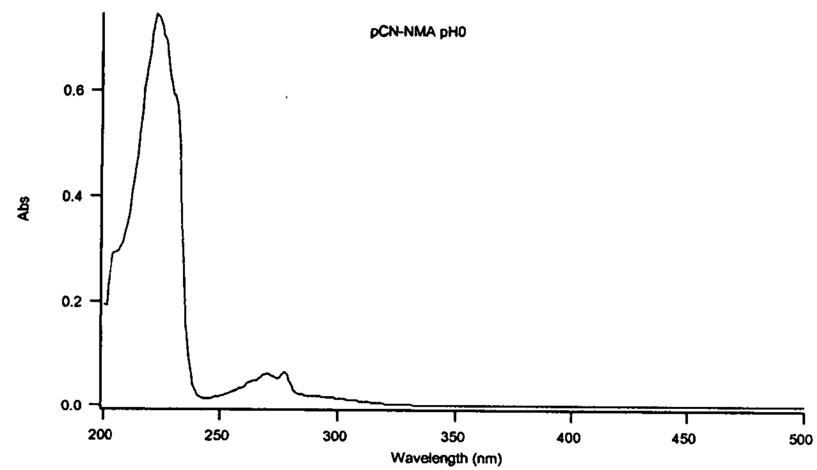


(left), 50uM Methemoglobin, 100 uM HNO donor, pH 7.4 50mM phosphate buffer; (right) same with added 1mM glutathione

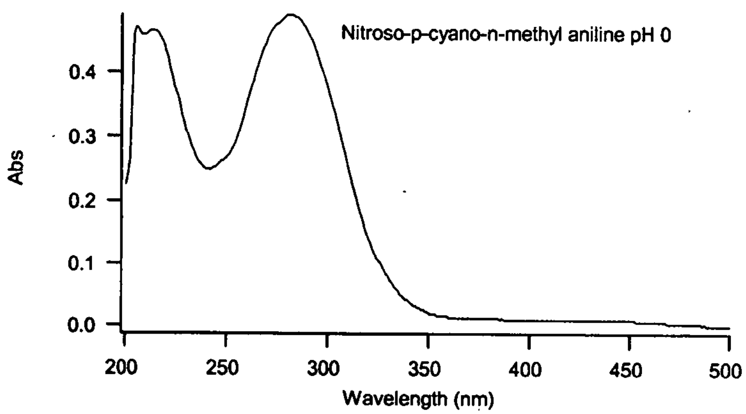
JHu Ref: 4390

Decomposition Assays

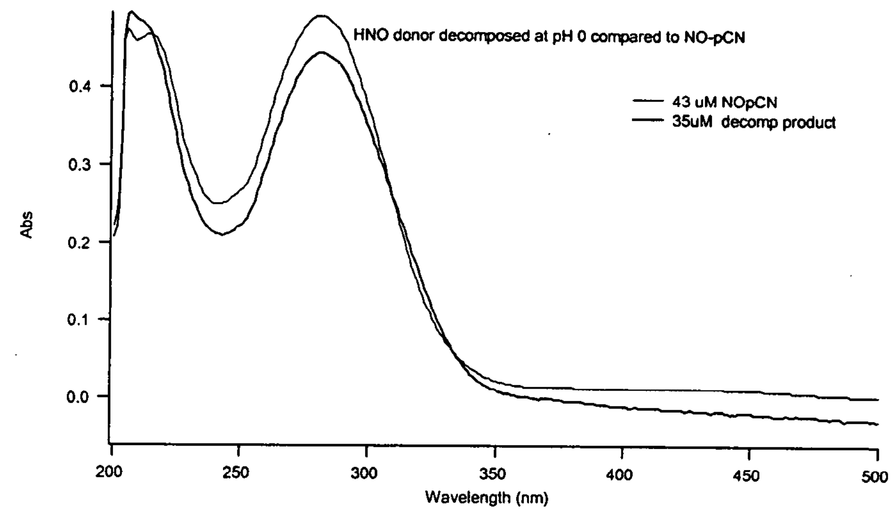




A. p-cyano-*N-methylaniline UV spectrum at pH0*



B. *N-Nitroso-p-cyano-N-methylaniline UV spectrum at pH0*



C. *Product of Decomposition at pH0 in an anaerobic environment. In red is the overlay of p-cyano-N-nitroso-N-methyl aniline UV spectrum at pH0.*

This assay shows that nitrosamine is formed during decomposition, a proposed product of decomposition of HNO/nitrosamine complexes

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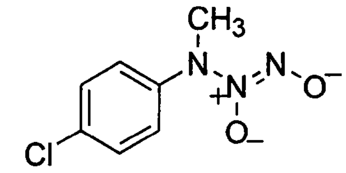
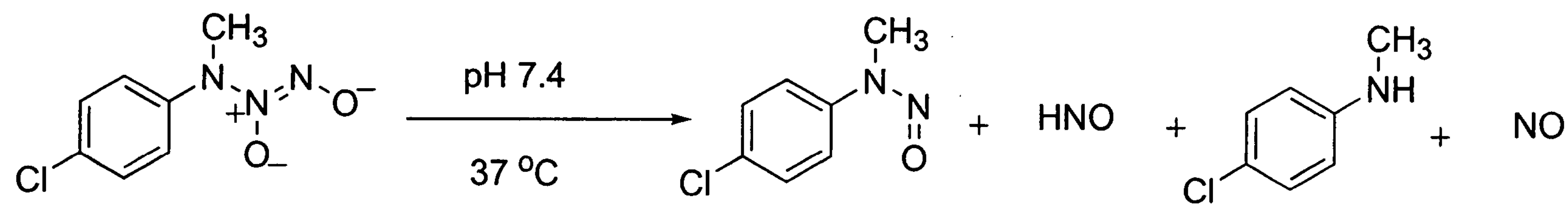
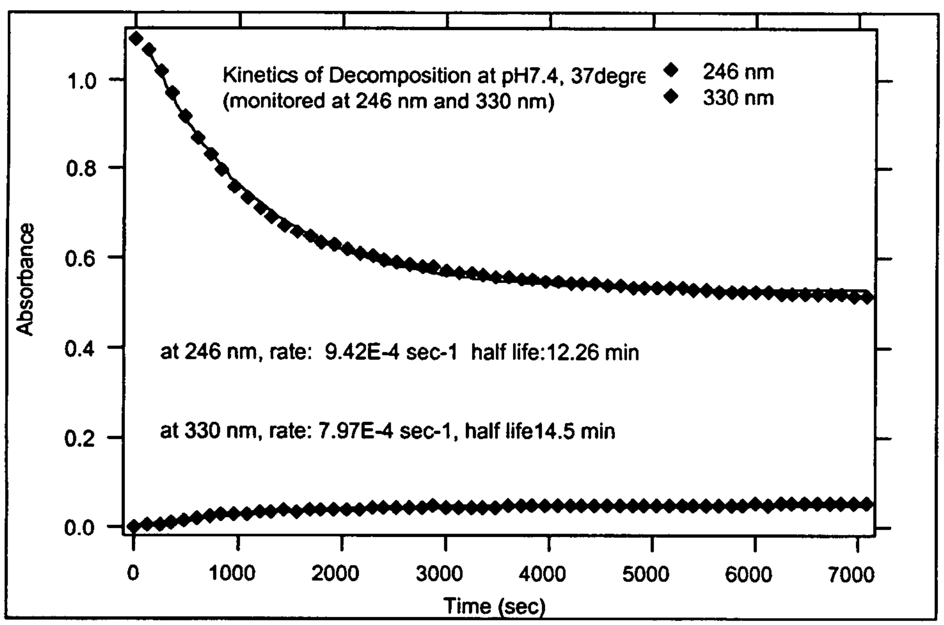
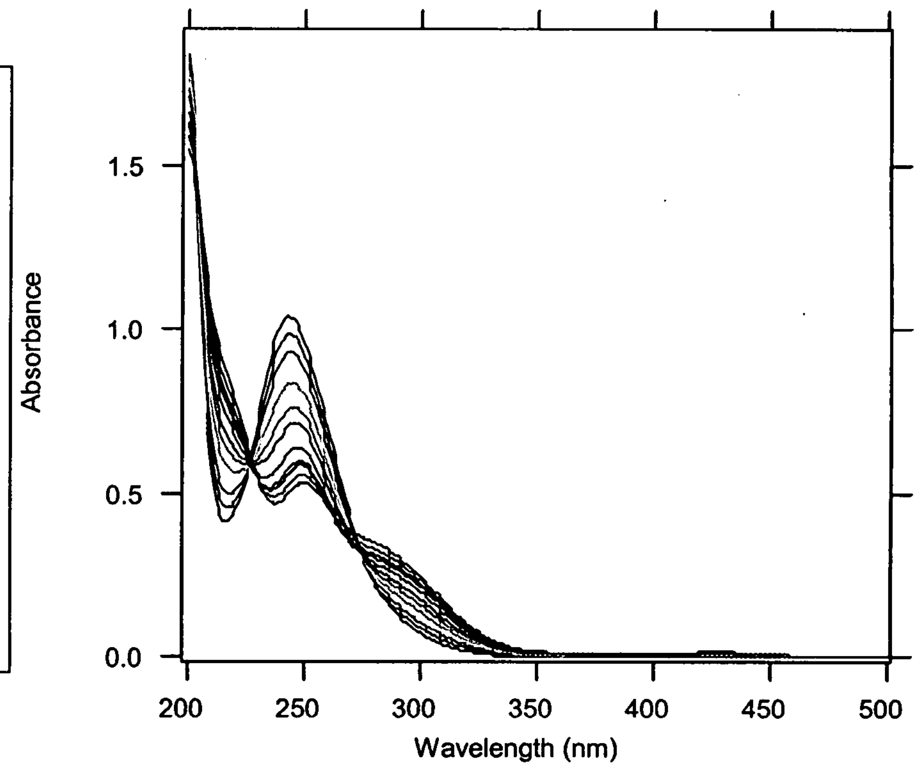
Thermal Decomposition of 



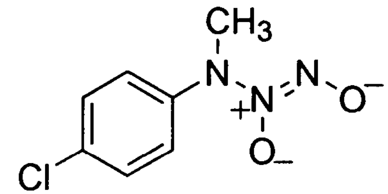




(left): Kinetics of decomposition at 37 degrees C, pH7.4, monitored at 246 nm (max absorbance of HNO/NO donor). (right): spectral data of the decay taken over a period of 2 hours.

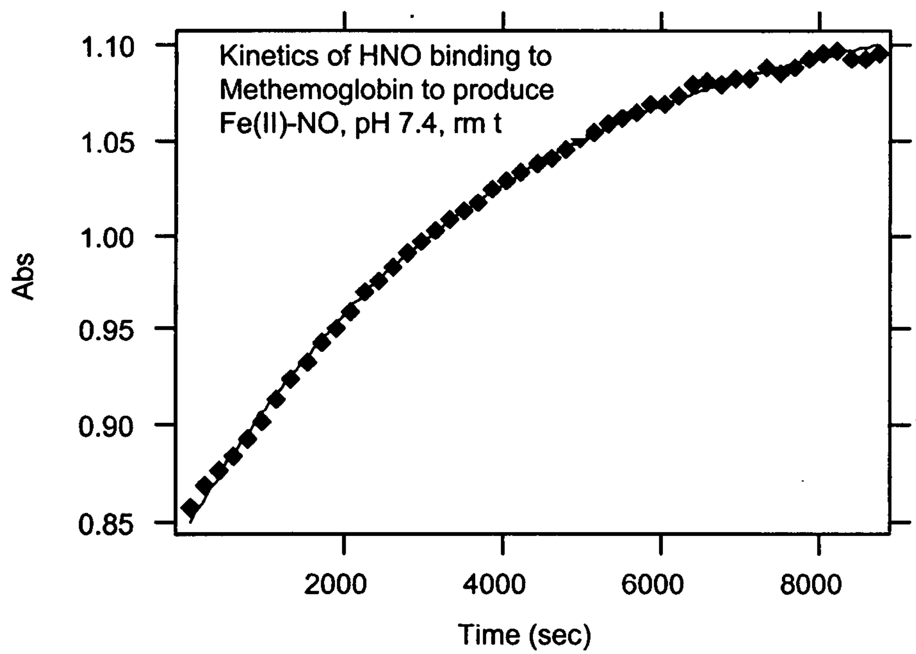
JHu Ref: 4390

Methemoglobin (Hb^+) Assays with

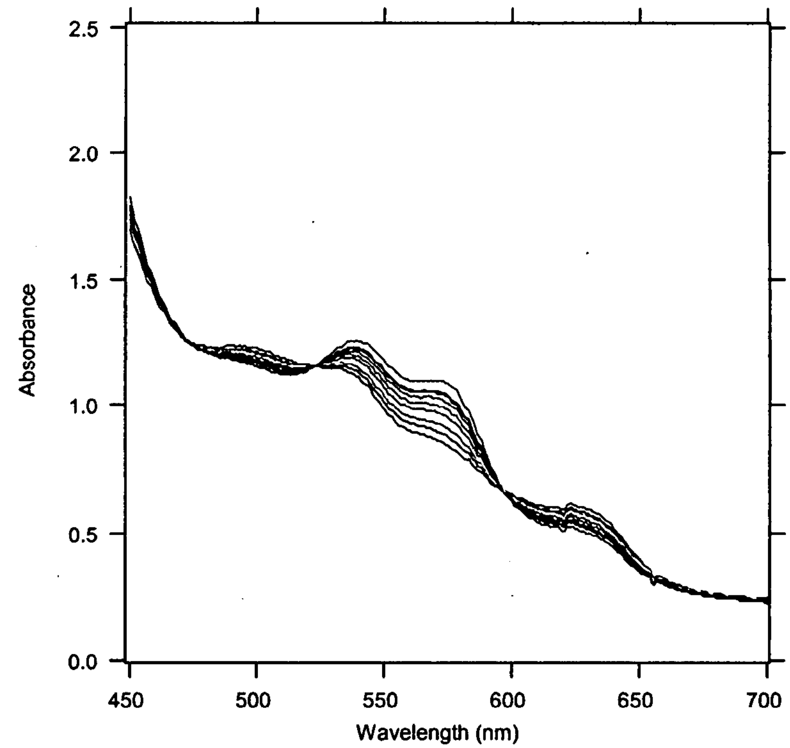


$$\mathrm{Fe(III) + HNO \longrightarrow Fe(II)\text{-}NO}$$

Kinetics of Hb^+ binding to HNO



Spectral Monitoring of Hb^+ binding to HNO



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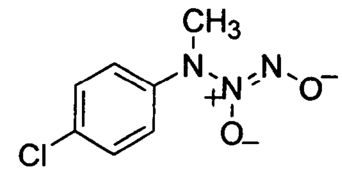
(left): Kinetics of Fe(II)-NO production at pH7.4, monitored at 572 nm, concentration of HNO donor: 100 uM and Methemoglobin 50 uM. The change in absorbance at 572 nm (E=13,000 $M^{-1}cm^{-1}$) is equal to .63 eq of HNO (right): spectral data taken over a period of 2 hours.

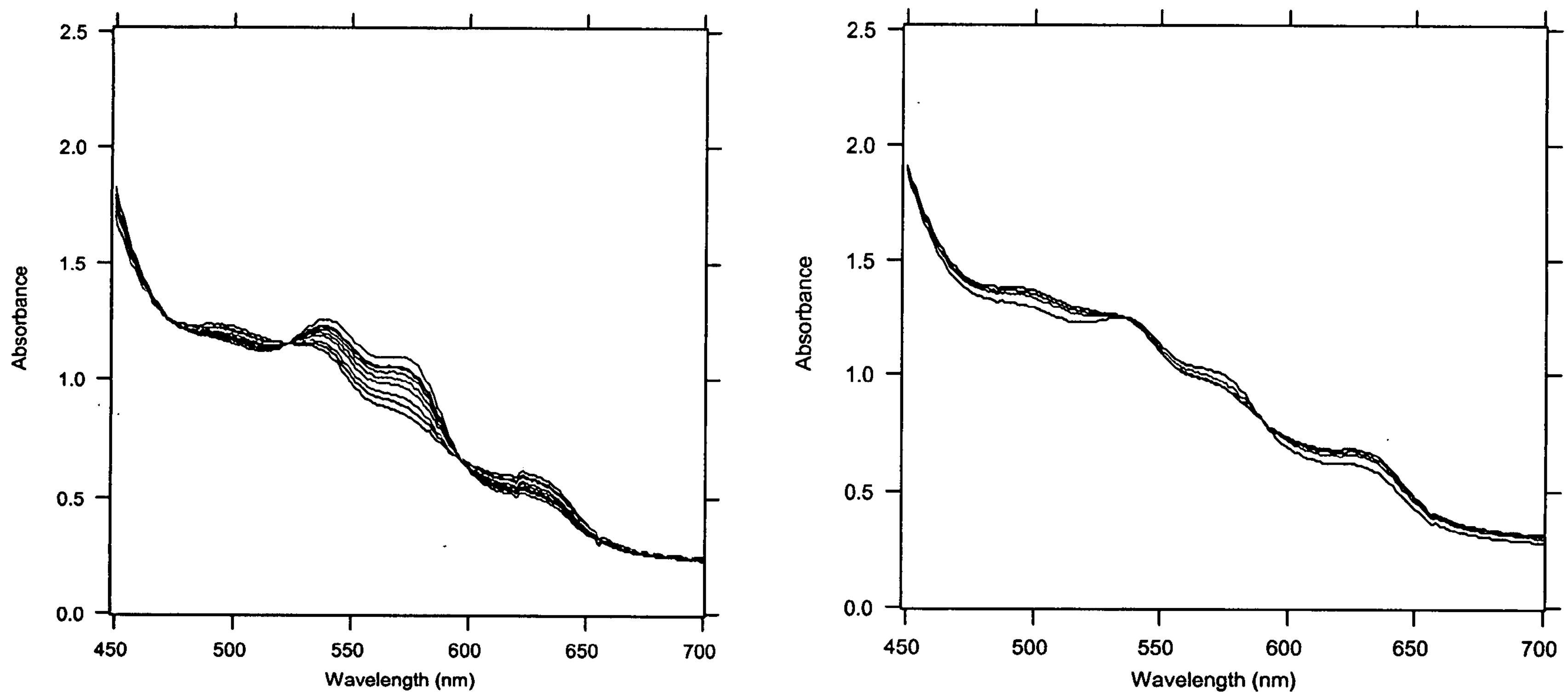
JHu Ref: 4390

Quenching Methemoglobin Assays with Glutathione



Angeli's Salt is a known HNO donor that releases one equivalent of HNO per molecule.

In the absence *In the presence*



(left), 50uM Methemoglobin, 100 uM HNO donor, pH 7.4, 50mM phosphate buffer; (right) same with added 1mM glutathione

Dog preparation and methods:

Male mongrel dogs (20 to 30 kg) were anesthetized with 1-2% Isoflurane after induction with sodium pentothal. The chest was opened via a lateral thoracotomy, and indwelling catheters (Tygon; Norton Plastics and Synthetic Division) secured in the right atrium (for drug infusion) and in the descending aorta (for pressure measurement). An indwelling high-fidelity micromanometer (P22, Konigsberg Instruments) was placed in the left ventricle (LV) through an apical stab. Two endocardial sonomicrometer crystals were placed at the cardiac base – from which a left ventricular antero-posterior internal dimension was generated. A coronary flow probe (Transonic) was placed at the proximal left circumflex coronary artery to measure coronary flow velocity. A pneumatic occluder was placed around the IVC to allow preload reduction for assessing PV relations. Pacing leads were attached to the left atrium for acute pacing during experimentation. After the chest was closed, catheters and leads were externalized to the midscapulae and protected by an external jacket. Analgesia (buprenorphine 0.3 mg/kg every 12 hours) was given in the immediate postoperative period as necessary, and antibiotics administered for the first 72 hr post-operative period. Dogs were allowed 10 days for recover prior to studies.

Studies were performed with animals supported in a sling apparatus, conscious, with all sensors connected to signal processors and custom software for displaying real-time pressure-dimension data. Hemodynamic measurements were performed at the constant atrial pacing rate (140 beats per minute). To identify the role of baroreflex activation, 10% (wt/vol) dextran was rapidly infused to restore chamber loading to baseline. Chronic heart failure (CHF) was induced by chronic rapid ventricular pacing at a rate of 210 beats per minute for 3 weeks followed by 240 beats per minute for 1 week.

Results:

In control dog. Compound A and Compound B were administrated to a healthy control dog at the dose of 2.5μg/kg/min. Table 1 shows the summary data. Both Compound A and Compound B increased load-independent contractility indexes (End-systolic elastance; Ees, +25.2% and +109.6%, respectively), and reduced preload (end-diastolic dimension, EDD; -11.1% and –12.9%, respectively) and after-load (total resistance, RT; -24.0% and –15.1%, respectively). But after volume loading, Compound A had no effect on myocardial contractility, while Compound B still enhanced contractility (Ees; -14.4% and +45.4%, respectively).

In CHF dog. Figure 1 shows representative P-D loops in a CHF hearts with compound B administration (1.25μg/kg/min) and volume restoration. EDD and systolic pressure both declined, whereas Ees was enhanced, denoted by its left shift and higher slope (middle). Even after EDD and systolic pressure was restored by volume loading, Ees was still enhanced (bottom). Table 2 provides summary data. Compound B reduced pre-load (EDD; -9.9%) and after-load (RT; -26.1%), and enhanced contractility (Ees; +70.6%). Positive inotropic effect was still observed (Ees; +33.5%) after volume restoration (EDD; -2.2%, end-systolic pressure; -4.6%).

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Table 1. Cardiovascular effects in control dog.

	Comound A(2.5μg/kg/min)			Comound B (2.5μg/kg/min)		
	before	after	+ volume loading	before	after	+ volume loading
Ees (mmHg/mm)	11.6	14.5	9.9	8.5	17.9	12.4
Tau (msec)	34.4	31.6	32.0	38.5	30.4	33.9
LVEDD (mm)	31.1	27.7	30.7	32.5	28.3	31.6
LVESD (mm)	23.6	20.7	22.3	23.4	20.0	21.6
LVESP (mmHg)	137.4	96.3	118.4	137.4	107.9	123.9
LVEDP (mmHg)	5.5	2.6	5.5	9.9	5.7	5.3
RT (mmHg/mm/sec)	7.3	5.6	5.6	6.1	5.2	5.0

Ees, end-systolic elastance; D_{EDD}, dP/dt-end-diastolic dimension relation; PRSW, prerecruitable stroke work; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; RT, total resistance.

Table 2. Compound B induced changes in control and CHF dog.

	Control		CHF	
	Comound B (2.5μg/kg/min)	+ volume loading	Comound B (1.25μg/kg/min)	+ volume loading
Ees (mmHg/mm)	+109.6%	+45.4%	+70.6%	+33.5%
Tau (msec)	-21.0%	-12.0%	-21.5%	-19.7%
LVEDD (mm)	-12.9%	-2.7%	-9.9%	-2.2%
LVESD (mm)	-14.3%	-7.4%	-11.5%	-6.5%
LVESP (mmHg)	-21.5%	-12.0%	-18.6%	-4.6%
LVEDP (mmHg)	-36.8%	-8.4%	-44.4%	-9.2%
RT (mmHg/mm/sec)	-15.1%	-18.7%	-26.1%	-35.6%

Ees, end-systolic elastance; D_{EDD}, dP/dt-end-diastolic dimension relation; PRSW, prerecruitable stroke work; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; RT, total resistance.

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Figure 1

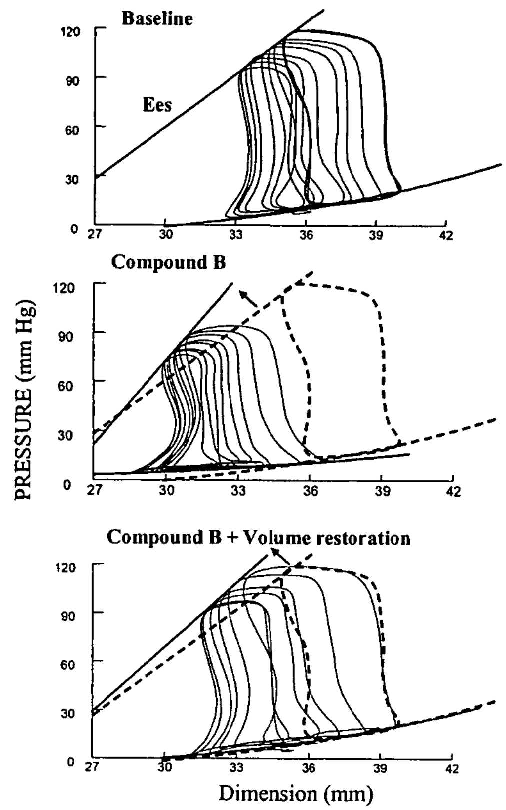
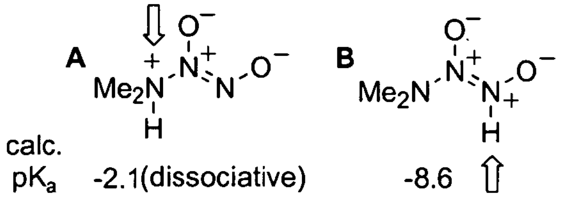
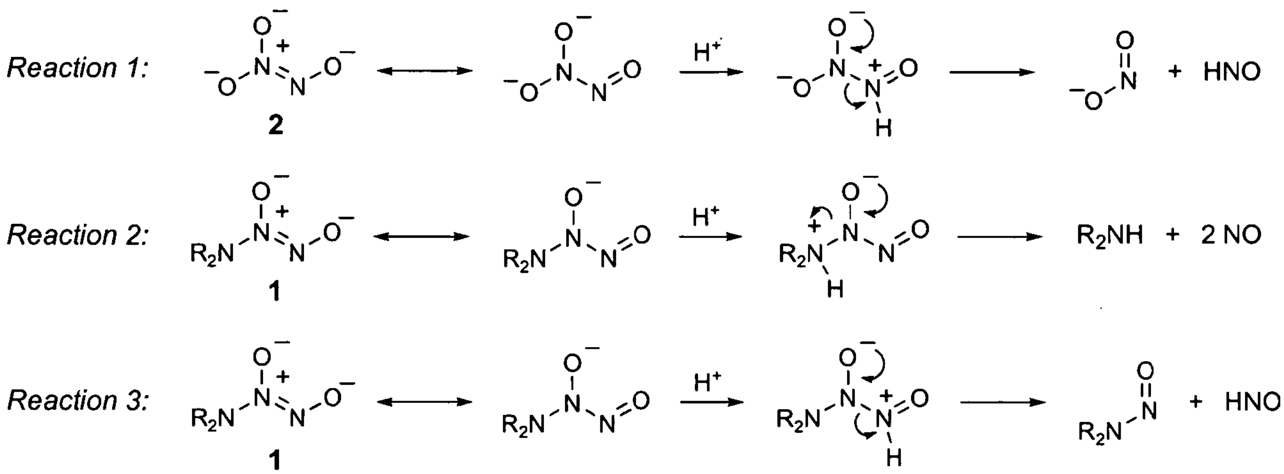


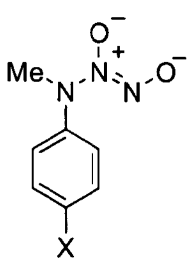
Figure 1 demonstrates efficacy of new HNO donor in the *in vivo* canine heart. The top panels display pressure-dimension loops and relations under baseline conditions. Upper line reflects contractile function. The middle panel displays results of infusion of the new HNO donor (Compound B) in the same animal. The leftward shift of the end-systolic pressure-dimension relation (line, upper left of loops) indicates positive contractile effect. This was accompanied by a decline in chamber preload volume (i.e. venodilation) (loops shift leftward as well). To minimize this effect, we infused volume to the animal restoring preload volume to the baseline level (lower panel). There is still a clear increase in contractile function (arrow) with Compound B. Thus, the new compound is a positive inotrope and venodilator in the conscious dog.

N-Methylaniline-based diazeniumdiolate derivatives as HNO donors.

Angeli's salt (**2**) and DEA/NO (**1**, R = Et) are both diazeniumdiolate derivatives; however, at physiological pH, Angeli's salt is an HNO donor (Reaction 1) and DEA/NO is an NO donor (Reaction 2). Why? The answer, of course, is related to the relative ease of protonation and subsequent barriers to decomposition from the relevant protonated species. *Prior to our work described below, all known secondary amine-based diazeniumdiolate derivatives have been demonstrated to be NO donors.* The mechanism of NO formation from the decomposition of dialkylamino diazeniumdiolates has very recently been examined computationally by Houk and co-workers.[1] These results are consistent with experiments[2] and indicate that the terminal oxygen is most basic, followed closely by the internal oxygen, but that protonation at these sites does not lead to decomposition. Protonation of the weakly basic amino nitrogen, however, leads to very rapid decomposition and NO generation. These calculations (see structures **A** and **B**) suggest that protonation leading to the products of Reaction 2 is favored over that leading to the products of Reaction 3 by approximately 6.5 pK_a units.






3

Consistent with, but independent of these computations, we reasoned that if we made protonation at the amino nitrogen very unfavorable, we could make Reaction 3 accessible for secondary amine diazeniumdiolate derivatives. Thus, we prepared *N*-methylaniline derivatives **3**, where X = H, Cl, and CN by treating a solution of the appropriate *N*-methylaniline derivative (1 g) in methanol (5 mL) with one equivalent of sodium methoxide (25% w/w in methanol) in a standard Parr hydrogenation bottle. The reaction vessel was purged with nitrogen and then saturated with excess NO. The reaction was allowed to stir at room temperature for 48 hours during which time the pressure of NO gas was maintained at approximately 40 psi. The product was isolated by filtration, washed with ethyl ether, dried under vacuum, and characterized by standard analytical methods. We examined the decomposition of derivatives **3** at 37 °C and pH 7.4. Organic products were quantified by UV-Vis, NMR, and HPLC analysis. NO was detected electrochemically using an inNO Measuring System with an amiNO-700 probe (Innovative Instruments) as we have reported previously.[3] HNO was measured by trapping with methemoglobin as has been described in the literature.[4] We also assayed for the presence of N_2O (the product of HNO dimerization and subsequent dehydration) by gas chromatography. As shown in the Table below, our results indicate that the decomposition products (and thus the relative contributions of Reactions 2 and 3) are strongly dependent on the *para*-substituent X. *Importantly, derivative 3 with X = CN represents the first secondary amine-based diazeniumdiolate that is a pure HNO donor.*

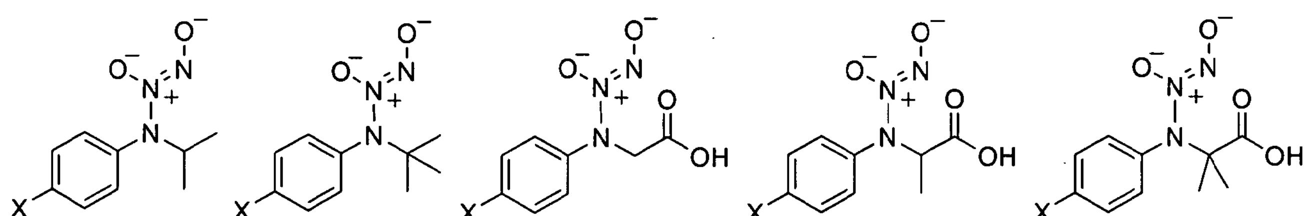
X	% Amine	% NO	% Nitrosamine	% HNO	N_2O ?	half-life (37 °C, pH 7.4)[b]
H	100	180	0	0	no	4 min
Cl	≥95	*a*	trace	*a*	*a*	12 min
CN	0	0	100	100	yes	11 min

a = not yet determined; b = for comparison, the half-life of Angeli's salt under these conditions is 2 min

The results for compounds **3** observed as a function of *para* substituent X are also consistent with Houk's calculations. As mentioned above, these calculations predict a pK_a difference of approximately +6.5 units (pK_a(**A**) – pK_a(**B**)) for the dimethylamino derivative. The pK_a's of protonated dimethylamine, *N*-methylaniline, *p*-chloro-*N*-methylaniline, and *p*-cyano-*N*-methylaniline are 10.8, 5.0, 4.5, and 2.2, respectively.[5] Thus, based on these pK_a's, Houk's calculations, and the assumption that the pK_a of structure **B** is unaffected by changes at the amine nitrogen, we estimate that the pK_a differences for the corresponding protonated forms **A** and **B** are +0.7, +0.2, and –2.1 for **3** with X = H, Cl, and CN, respectively. This analysis predicts that the pK_a of the protonated secondary amine from which the diazeniumdiolate is made should be a good indicator of whether the resulting diazeniumdiolate will be an NO or an HNO donor: *Any diazeniumdiolate made from an amine whose protonated form has a pK_a below 3 or 4 should be an HNO donor.*

Although diazeniumdiolate **3** with X = CN is a pure HNO donor, an obvious practical drawback is that the by-product of its decomposition is a potentially carcinogenic nitrosamine (Reaction 3). The carcinogenicity of nitrosamines is thought to arise via metabolic activation by cytochrome P450 enzymes to give α-hydroxynitrosamines that undergo subsequent reactions resulting in DNA alkylation.[6,7] Lijinsky and co-workers have found that replacement of even a single α-hydrogen with a methyl group greatly reduces nitrosamine carcinogenicity and that total blockage of any potential oxidation site leads to a non-carcinogenic nitrosamine (e.g., *N*-nitroso-2,2,6,6-tetramethylpiperidine).[6] In addition, carboxylic acid substitution also greatly reduces or eliminates (e.g., nitrosoproline) carcinogenicity, presumably the result of rapid excretion before enzymatic oxidation can occur.[6]

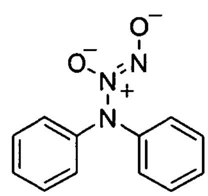
Based on these observations, obvious candidate diazeniumdiolates **4** – **8** are shown below with X = CN or NO_2.



4 **5** **6** **7** **8**

Diphenylamine-based -based diazeniumdiolate derivatives as HNO donors.

As an alternative to diazeniumdiolates based on derivatives of *N*-methylaniline, diazeniumdiolates based on commercially available diphenylamine (**9**) and the corresponding *p*-dicarboxylate (**10**) are also predicted to be HNO donors. The pK_a of diphenylammonium is 0.78 in water, which suggests, by our hypothesis, that a diazeniumdiolate based on this amine should be an HNO donor. Additionally, *N*-nitrosodiphenylamine contains no α-hydrogens, and therefore, is expected to be a non-carcinogenic byproduct. *N*-nitrosodiphenylamine has been shown to be non-carcinogenic in mice[6] and has also recently been shown to be non-mutagenic by the Ames test.[8,9] As mentioned above, the addition of carboxylic acid substitution has been shown to reduce carcinogenicity, thus dicarboxylate compound **10** should be a good candidate as an HNO donor for *in vivo* use.



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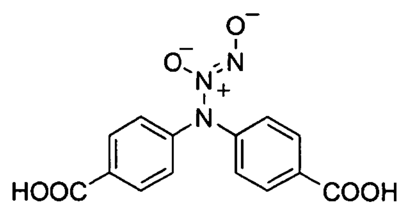

10

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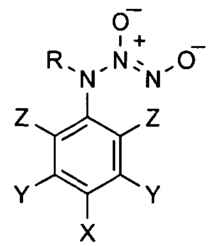
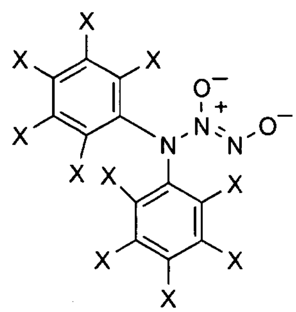
Future Derivatives



where R is H, a primary, secondary, or tertiary alkyl group, or an aromatic group; X is an electron-withdrawing substituent (e.g., halogen, CN, NO_2, CO_2H, CO_2R, CF_3); Z is H, an alkyl group, or an electron-withdrawing substituent (e.g., halogen, CN, NO_2, CO_2H, CO_2R, CF_3); Y is H, alkyl, or CO_2H.



where X is H, alkyl, CO_2H, or an electron-withdrawing substituent.

Diazeniumdiolates that can be synthesized from any amine (R_2NH) whose protonated form ($R_2NH_2^+$) has a pK_a below 4.5 are predicted to be nitroxyl (HNO) donors.